

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188
<p>Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.</p>			
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED	
	February 1995	FINAL	15/07/91 - 31/12/94
4. TITLE AND SUBTITLE	EVOLUTION OF REGULATORY GENES GOVERNING BIODEGRADATION IN <u>ACINETOBACTER CALCOACETICUS</u>		5. FUNDING NUMBERS
6. AUTHOR(S)	L. Nicholas Ornston, Ph.D.		DAAL03-91-G-0227
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	<p>Yale University Department of Biology Kline Biology Tower - Room 750 P.O. Box 208103 New Haven, CT 06520-8103</p>		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	<p>U.S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211</p>		10. SPONSORING/MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES	<p>The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.</p>		
12a. DISTRIBUTION/AVAILABILITY STATEMENT	Approved for public release; distribution unlimited.		
12b. DISTRIBUTION CODE			
13. ABSTRACT (Maximum 200 words)	<p>The <i>Acinetobacter calcoaceticus</i> <i>pca-qui-pob</i> supraoperonic gene cluster encodes bacterial enzymes that metabolize aromatic and hydroaromatic compounds in the environment. Our investigation is directed to understanding how mutation, gene rearrangement and selection contributed to evolution of the transcriptional controls exercised over genes in the cluster. The complete nucleotide sequence of the 18 kbp gene cluster has been determined, and genetic manipulations have been used to explore mechanisms contributing to expression of the genes. The results reveal that structural gene expression is governed by complex interactions between the products of different regulatory genes some of which share common ancestry. Additional controls appear to be exercised by compartmentation of some catabolic enzymes outside the inner cell membrane. Recombination appears to have made a major contribution to the evolution of existing control mechanisms, and their maintenance may be influenced by continuing recombination. Contributions of recombination to mutation and repair are under investigation as are specific molecular mechanisms underlying transcriptional controls.</p>		
14. SUBJECT TERMS	mutation, repair, recombination, gene rearrangement, transcription, compartmentation, inner cell membrane		15. NUMBER OF PAGES 9
16. PRICE CODE			
17. SECURITY CLASSIFICATION OF REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UL

19950308 047

EVOLUTION OF REGULATORY GENES GOVERNING  
BIODEGRADATION IN ACINETOBACTER CALCOACETICUS

FINAL REPORT

L. N. Ornston

February 22, 1995

Covering Dates 15/07/91 - 31/12/94

U.S. Army Research Office  
P.O. Box 12211  
Research Triangle Park, NC 27709-2211

Grant Number DAAL03-91-G-0227/28964-LS

Accesion For	
NTIS	CRA&I <input checked="" type="checkbox"/>
DTIC	TAB <input type="checkbox"/>
Unannounced <input type="checkbox"/>	
Justification .....	
By .....	
Distribution /	
Availability Codes	
Dist	Avail and/or Special
A-1	

Yale University  
Biology Department  
Kline Biology Tower  
P.O. Box 208103  
New Haven, CT 06520-8103

Approved for public release;

Distribution unlimited.

The views, opinions, and/or findings contained in this report are those of the author and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

The *Acinetobacter calcoaceticus* *pca-qui-pob* supraoperonic gene cluster encodes bacterial enzymes that metabolize aromatic and hydroaromatic compounds in the environment. Our investigation is directed to understanding how mutation, gene rearrangement and selection contributed to evolution of the transcriptional controls exercised over genes in the cluster. The complete nucleotide sequence of the 18 kbp gene cluster has been determined, and genetic manipulations have been used to explore mechanisms contributing to expression of the genes. Several surprises have emerged from this investigation.

1. The *catIJF* and *pcaIJF* genes, separated by about 270 kbp of DNA in the *Acinetobacter calcoaceticus* chromosome, exhibit 99% nucleotide sequence identity extending over 2.2 kbp. Earlier evidence had indicated that *catIJF* could serve as template for repair of mutations in *pcaIJF*. This process has been explored in greater detail. The repair has been shown to be mediated by gene conversion, and *pcaIJF* can mediate repair of mutations in *catIJF*. This process of ongoing genetic exchange may contribute to conservation of codon usage patterns which make *catIJF* and *pcaIJF* unusual among *A. calcoaceticus* genes. Nucleotide tracts extending up to 881 base pairs were shown to be transferred by during repair (Kowalchuk et al., 1994, 1995), and the process was shown to depend upon *recA* (Gregg-Jolly and Ornston, 1994).

2. The ease with which *recA* mutations can be introduced into the *A. calcoaceticus* chromosome (Gregg-Jolly and Ornston, 1994) makes it possible to explore interaction of mutant and wild type alleles in the absence of recombination. Thus it was possible to demonstrate that *pobR*, the transcriptional activator of *pobA* (the structural gene for *p*-hydroxybenzoate hydroxylase), participates in regulation of its own synthesis. The molecular basis for transcriptional controls in the *pobR-pobA* intergenic region was explored (DiMarco and Ornston, 1994). Downstream from *pobR* and expressed in the same transcript is *pobS*, an apparent repressor of *pobA* expression. It is not clear why a transcriptional repressor should be expressed directly downstream from a transcriptional activator. The physiological functions of *pobS* are still under examination.

3. Possibilities for introduction of a *recA* null mutation into the *A. calcoaceticus* chromosome also made it possible to explore contributions of specified DNA fragments to quinate and shikimate catabolism. The *quiA* gene encodes a transmembrane oxidase that converts quinate to dehydroquinate and shikimate to dehydroshikimate. This gene and other genes associated with the catabolism of quinate and shikimate to protocatechuate lie between *pcaG* and *pobS* in the *A. calcoaceticus* chromosome (Elsemore and Ornston, 1994).

4. Conversion of dehydroquinate to dehydroshikimate is a necessary step in the biosynthesis of aromatic amino acids. In enteric bacteria, the biosynthetic gene is encoded by *aroD*. Catabolic conversion of dehydroquinate to dehydroshikimate is mediated by the enzyme encoded by *quiB*, and an *A. calcoaceticus* DNA fragment containing *quiB* was identified on the basis of its ability to complement a null mutation in *E. coli aroD*. Nucleotide sequence analysis revealed that *A. calcoaceticus* *quiB* resembles the biosynthetic *aroD* of other organisms and is unlike the genes with the catabolic *quiB* function. Therefore gene rearrangement appears to have placed a biosynthetic gene in a catabolic cluster during the evolution of *A. calcoaceticus*.

5. Further characterization of genes in the *qui* region revealed *quiX* which encodes a porin-like protein. Introduction of null mutations into the gene do not prevent growth with quinate or shikimate, but the possibility that the protein contributes to growth in the presence of low substrate concentrations has not been explored.

6. Nucleotide sequence similarities suggest a transport function for *pcaK*, another gene within the *pca-qui-pob* cluster. The physiological contribution of *A. calcoaceticus* *pcaK* is under investigation.

7. Procedures for selection of null mutants in *pob* and *pca* genes were developed and applied to a detailed examination of mutations in *pcaH,G*, genes encoding the homologous protein subunits of protocatechuate 3,4-dioxygenase. This investigation (Gerischer and Ornston, 1995) revealed regions of DNA in which secondary structures formed between slipped DNA strands may predispose specific nucleotides to genetic alteration. Mutants isolated in this study facilitated understanding of a promoter region directly upstream of the *pca* operon and led to identification of *pcaU*, a divergently transcribed activator of the *pca* operon. The *pcaU* and *pobR* transcriptional activators are closely related members of a sparsely represented gene family. Despite their similarity, the activators are highly selective in the genes that they control. Exploration of *pcaU* and the mechanisms by which it exerts control are continuing.

8. Analysis of spontaneous *pcaH,G* mutants (Gerischer and Ornston, 1995) revealed an insertion sequence, IS1236, which was responsible for about 10% of the sequenced mutations. Insertion of this genetic element in *pcaH,G* was somewhat imprecise in the sense that the length of repetition of flanking nucleotides varied considerably. Remarkably, IS1236 accounts for 5 out of 6 mutations isolated thus far in *pobR*, and in every case the insertion is flanked by a precise 3 base pair repetition. Thus both the frequency and the mode of insertion appear to be influenced by the target DNA. Investigation of IS1236 continues.

7. A longstanding investigation was brought to conclusion with publication of the nucleotide sequence of the *catR,BCA* region from the *Pseudomonas putida* (Houghton et al., 1995). This work is of particular interest because it suggests that nucleotide sequences may have been exchanged between *catC* and *catA* after transposition introduced the latter gene directly downstream from *catA*. Possible participants in the slippage of DNA strands that would be required for intergenic sequence exchange emerged from identification of REP (Repetitive Extragenic Palindrome)-like genetic elements at different locations in the *catR,BCA* cluster from two different fluorescent *Pseudomonas* strains.

Our present investigations are directed towards further characterization of transcriptional controls exercised over genes in the *pca-qui-pob* supraoperonic cluster. Transcription-terminating omega elements and *lacZ* reporter genes have been introduced at different loci within the cluster, and these constructs will give a indication of the sites where transcriptional control is exercised. More specific information will be obtained by identifying sites of transcriptional initiation.

In an effort to analyse the physiological basis for supraoperonic clustering, the *catA* gene has been transposed from its normal locus within the *ben-cat* cluster to a position within *pobA*. The strain containing the transposition grows with benzoate (a physiological process requiring expression of *catA*) only if *p*-hydroxybenzoate (which elicits expression of the *pobA* transcript is present). In principle, selection for growth with benzoate in the absence of *p*-hydroxybenzoate should yield mutants expressing *pobA-catA* constitutively, but such mutants have not been obtained. This failure may be a reflection of the extremely tight *pobA* transcriptional controls which are under further investigation. As part of this study, an alternative approach (selection for elevated expression of *folA* after it had been placed downstream from *pobA* on a plasmid) led to heightened *pobA* expression in *E. coli* (Fernandez et al., submitted for publication).

Publications

1. **Neidle, E. L., C. Hartnett, L. N. Ornston, A. Bairoch, M. Rekik, and S. Harayama.** 1991. Nucleotide sequences of the Acinetobacter calcoaceticus benABC genes for benzoate 1,2-dioxygenase reveal evolutionary relationships among multicomponent oxygenases. *J. Bacteriol.* **173**:5385-5395.
2. **Harayama, S., M. Rekik, A. Bairoch, E. L. Neidle, and L. N. Ornston.** 1991. Potential DNA slippage structures acquired during evolutionary divergence of Acinetobacter calcoaceticus chromosomal benACB and Pseudomonas putida TOL pWWO plasmid xylXYZ, genes encoding benzoate dioxygenases. *J. Bacteriol.* **173**:7540-7548.
3. **Averhoff, B., L. Gregg-Jolly, D. Elsemore and L. N. Ornston.** 1992. Genetic analysis of supraoperonic clustering in Acinetobacter calcoaceticus. *J. Bacteriol.* **174**:200-204.
4. **Neidle, E., C. Hartnett, L. N. Ornston, A. Bairoch, M. Rekik and S. Harayama.** 1992. cis-Diol dehydrogenases encoded by the TOL pWWO) plasmid xylL gene and the Acinetobacter calcoaceticus chromosomal benD gene are members of the short-chain alcohol dehydrogenase superfamily. *Eur. J. Biochem.* **204**:113-120.
5. **DiMarco, A. A., B. Averhof, E. E. Kim, and L. N. Ornston.** 1993. Evolutionary divergence of pobA, the structural gene for p-hydroxybenzoate hydroxylase, in an Acinetobacter calcoaceticus strain well-suited for genetic analysis. *Gene* **125**:25-33.
6. **Schlömann, M. J., K.-L. Ngai, L. N. Ornston, and H.-J. Knackmuss.** 1993. Dienelactone hydrolase from Pseudomonas cepacia. *J. Bacteriol.* **175**:2994-3001.
7. **DiMarco, A. A., B. Averhoff, and L. N. Ornston.** 1993. Identification of the transcriptional activator pobR, and characterization of its role in the expression of pobA, the structural gene for p-hydroxybenzoate hydroxylase in Acinetobacter calcoaceticus. *J. Bacteriol.* **175**:4499-4506.
8. **Shanley, M. S., A. Harrison, R. E. Parales, G. Kowalchuk, D. J. Mitchell and L. N. Ornston.** 1994. Unusual G+C content and codon usage in catIJF, a segment of the ben-cat supra-operonic cluster in the Acinetobacter calcoaceticus chromosome. *Gene* **138**:59-65.
9. **Hartnett, G. B. and L. N. Ornston.** 1994. Acquisition of apparent DNA slippage structures during extensive evolutionary divergence of pcaD and catD, genes for enzymes with identical catalytic activities in Acinetobacter calcoaceticus. *Gene* **142**:23-29.

10. Gregg-Jolly, L. A. and L. N. Ornston. 1994. Properties of Acinetobacter calcoaceticus recA and its contribution to intracellular gene conversion. *Mol. Microbiol.* **12**:985-982.
11. DiMarco, A. A. and L. N. Ornston. 1994. Regulation of p-hydroxybenzoate hydroxylase synthesis by PobR bound to an operator in Acinetobacter calcoaceticus. *J. Bacteriol.* **176**:4277-4284.
12. Kowalchuk, G. A., Hartnett, G. B., Benson, A., Houghton, J. E., Ngai, K. L. and L. N. Ornston. 1994. Contrasting patterns of evolutionary divergence within the Acinetobacter calcoaceticus pca operon. *Gene* **146**:23-30.
13. Ehrt, S., L. N. Ornston and W. Hillen. 1994. RpoN ( $\sigma^{54}$ ) is required for conversion of phenol to catechol in Acinetobacter calcoaceticus. *J. Bacteriol.* **176**: 3493-3499.
14. Elsemore, D., and L. N. Ornston. 1994. The pca-pob supraoperonic cluster of Acinetobacter calcoaceticus contains quiA, the structural gene for quinate/shikimate dehydrogenase. *J. Bacteriol.* **176**:7659-7666.
15. Gerischer, U., and L. N. Ornston. 1995. Spontaneous mutations in pcaH,G, structural genes for protocatechuate 3,4-dioxygenase in Acinetobacter calcoaceticus. *J. Bacteriol.*, In Press.
16. Kowalchuk, G. A., L. A. Gregg-Jolly, and L. N. Ornston. 1994. Nucleotide sequences transferred by gene conversion in the bacterium Acinetobacter calcoaceticus. *Gene*, In Press.
17. Houghton, J. E., T. M. Brown, A. J. Appel, E. J. Hughes, and L. N. Ornston. 1995. Discontinuities in the evolution of Pseudomonas putida cat genes. *J. Bacteriol.* **177**:401-412.

Personnel

David A. Elsemore, Ph.D., 1995

Anthony A. DiMarco

George A. Kowalchuk, Ph.D., 1994

Ulrike Gerischer

Nicholas Ornston

Inventions

None

## REPORT OF INVENTIONS AND SUBCONTRACTS

Pursuant to "Patent Rights" - Contract Clause (See Instructions on Reverse Side.)

Public reporting burden for this collection of information is estimated to average 5 minutes per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0197). Washington, DC 20503.

Form Approved  
OMB No. 0704-029  
Expires Jan 30, 1993

1a. NAME OF CONTRACTOR/SUBCONTRACTOR <b>Yale University</b>		c. CONTRACT NUMBER <b>DAAL03-91-G-0227</b>		c. CONTRACT NUMBER <b>DAAL03-91-G-0227</b>		3. TYPE OF REPORT (X one) <input checked="" type="checkbox"/> a. FINAL <input type="checkbox"/> b. INTERIM	
b. ADDRESS (Include ZIP code) <b>12 Prospect Place New Haven CT 06511</b>		d. AWARD DATE (YYMMDD) <b>910715</b>		b. ADDRESS (Include ZIP code) <b>12 Prospect Place New Haven CT 06511</b>		d. AWARD DATE (YYMMDD) <b>910715</b>	
2a. NAME OF GOVERNMENT PRIME CONTRACTOR <b>Same</b>		2b. NAME OF GOVERNMENT PRIME CONTRACTOR <b>Same</b>		4. REPORTING PERIOD (YYMMDD) <b>a. FROM 910715 b. TO 911231</b>			

5. "SUBJECT INVENTION(S)" REQUIRED TO BE REPORTED BY CONTRACTOR / SUBCONTRACTOR (If "None," so state) <b>None</b>		SECTION I - SUBJECT INVENTIONS					
a.	b.	c.		d.		e.	
NAME(S) OF INVENTOR(S) (Last, First, MI)		TITLE OF INVENTION(S)		DISCLOSURE NO., PATENT APPLICATION SERIAL NO. OR PATENT NO.		ELECTION TO FILE PATENT APPLICATIONS	
						(1) United States      (2) Foreign (a) Yes      (b) No      (a) Yes      (b) No	
						(1) Yes      (2) No	

1 EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR / SUBCONTRACTOR		9 ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) Name of Inventor (last, first, MI)		(2) Foreign Countries of Patent Application	
(2) (a) Name of Inventor (last, first, MI)		(1) Title of Invention	
(b) Name of Employer		(2) Name of Employer	
(3) Address of Employer (include Zip Code)		(4) Address of Employer (include Zip Code)	

<b>SECTION II - SUBCONTRACTS (Containing a "Patent Rights" clause)</b>					
<b>b. NAME OF SUBCONTRACTOR(S)</b>		<b>b.</b> ADDRESS (Include ZIP Code)	<b>c.</b> SUBCONTRACT NO(S)	<b>d. DEAR "PATENT RIGHTS"</b>	<b>f. SUBCONTRACT DATES (YY/MM/DD)</b>
			(1) Clause Number	(2) Date (YY/MM)	(1) Award (2) Estimated Completion
e. DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S)					
None					
<b>6. SUBCONTRACTS AWARDED BY CONTRACTOR / SUBCONTRACTOR (if "None," so state)</b>					

SECTION III - CERTIFICATION				
7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR		(Not required if	Small Business or	Non-Profit organization) (X appropriate box)
a. NAME OF AUTHORIZED CONTRACTOR / SUBCONTRACTOR OFFICIAL (last, first, M.I.)		c. I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported		
Suzanne K. Palmer, Ph.D., Director				
b. TITLE	Grant and Contract Administration	d. SIGNATURE	e. DATE SIGNED	
		<i>A. M. O.</i>		<i>Feb. 1, 1991</i>